

Final Report

Study Title:

Algal Growth Inhibition Study

Author:

Koji Baba

Study Completed on:

July 22, 2010

Test Facility:

Nisso Chemical Analysis Service Co., Ltd. (NCAS)

Odawara Laboratory

345 Takada, Odawara, Kanagawa 250-0216, Japan

Sponsor:

Study Number:

NCAS 10-065

TRUE COPY OF ORIGINAL

6/5/12

Masato Sugawara

GLP Compliance Statement

Study No.: NCAS 10-065

Study title: Algal Growth Inhibition Study

This study was carried out in accordance with the following good laboratory practice regulation;

Standard for the test facility conducting tests concerning new chemical substances, etc. (Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, No. 1121003, November 21, 2003; Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry, No.3, November 17, 2003; Environmental Policy Bureau, Ministry of the Environment, No. 031121004) The latest amendment: July 4, 2008.

The final report was prepared faithfully and consistently with the raw data obtained.

Study Director:	(signature) _____	(seal) _____	July 22, 2010 _____
	Koji Baba		Date
	Nisso Chemical Analysis Service Co., Ltd.		
	Odawara Laboratory		

The original signature page of GLP Compliance Statement follows on page 3.

The original GLP Compliance Statement

The English translation of GLP Compliance Statement appears on page 2.

GLP 適合陳述書

試験番号： NCAS 10-065

試験名： の藻類に対する生長阻害試験

この試験は、「新規化学物質等に係る試験を実施する試験施設に関する基準について」平成 15 年 11 月 21 日 薬食発第 1121003 号、平成 15・11・17 製局第 3 号、環保企発第 031121004 号（最終改正 平成 20 年 7 月 4 日）に従って実施した。

この試験は、ここに述べられた方法により行われ、この最終報告書は試験実施により得られた生データを正確に反映したものである。

試験責任者： 馬場 康司  2010 年 7 月 22 日
馬場 康司
(株) 日曹分析センター 小田原事業所

Quality Assurance Statement

Study Number: NCAS10-065

Study Title: : Algal Growth Inhibition Study

Quality Assurance inspections of the study referred above were conducted according to the appropriate GLP regulations and the standard operating procedures (SOPs) of the Quality Assurance Unit (QAU). The results of the inspections were reported to the study director and the facility management on the following dates.

Items inspected	Dates (Month/Day/Year)		
	Inspected	Reported to	
		Study Director	Management
Protocol	5/24/2010	5/24/2010	5/24/2010
Experimental procedures			
• Acquisition and cultivation of algae	6/7/2010	6/10/2010	6/10/2010
• Preparation of the test solutions	5/25, 28/2010	5/28/2010	5/28/2010
• Exposure of algae to the test solutions	5/25, 28/2010	5/28/2010	5/28/2010
• Exposure of algae to the test solutions (Re-experiment)	6/7, 10/2010	6/10/2010	6/10/2010
• Measurement of the cell density	5/26, 28/2010	5/28/2010	5/28/2010
• Measurement of the cell density (Re-experiment)	6/10, 14/2010	6/14/2010	6/14/2010
• Analytical sampling and treatment	5/25, 28/2010	5/28/2010	5/28/2010
• LC/MS/MS analysis	5/25, 28/2010	5/28/2010	5/28/2010
• Microscopic observation(Re-experiment)	6/10, 14/2010	6/14/2010	6/14/2010
Raw Data	7/14 - 20/2010	7/20/2010	7/20/2010
Draft Report	7/14 - 20/2010	7/20/2010	7/20/2010
Final Report	7/22/2010	7/22/2010	7/22/2010

The QAU found that the study was performed according to the protocol and SOPs, the reported methods and procedures were actually used, and the results accurately reflect the recorded data.

QAU Manager: _____ (signature) _____ (seal) _____ (July 22, 2010)
 Ken Watabe
 Nisso Chemical Analysis Service Co., Ltd.

The original signature page of Quality Assurance Statement follows on page 5.

The original Quality Assurance Statement

The English translation of Quality Assurance Statement appears on page 4.

信頼性保証書

試験番号： NCAS 10-065

試験名： の藻類に対する
生長阻害試験

上記試験の信頼性保証の監査または査察を適用 GLP および信頼性保証部門（QAU）の SOP に基づいて実施した。監査または査察の結果は、以下の日付で試験責任者および運営管理者に報告した。

監査または査察項目	日付（月/日/年）		
	監査または 査察日	報告日	
		試験責任者	運営管理者
試験計画書	5/24/2010	5/24/2010	5/24/2010
実験操作			
・ 藻の入手、培養と継代	6/7/2010	6/10/2010	6/10/2010
・ 試験溶液の調製	5/25, 28/2010	5/28/2010	5/28/2010
・ 試験溶液への暴露	5/25, 28/2010	5/28/2010	5/28/2010
・ 試験溶液への暴露（再実験）	6/7, 10/2010	6/10/2010	6/10/2010
・ 細胞濃度の測定	5/26, 28/2010	5/28/2010	5/28/2010
・ 細胞濃度の測定（再実験）	6/10, 14/2010	6/14/2010	6/14/2010
・ 分析試料の採取と処理	5/25, 28/2010	5/28/2010	5/28/2010
・ LC/MS/MS 分析	5/25, 28/2010	5/28/2010	5/28/2010
・ 藻類の顕微鏡観察（再実験）	6/10, 14/2010	6/14/2010	6/14/2010
生データ	7/14-20/2010	7/20/2010	7/20/2010
報告書草案	7/14-20/2010	7/20/2010	7/20/2010
最終報告書	7/22/2010	7/22/2010	7/22/2010

QAU は、この試験が試験計画書および SOP に従って行われ、報告された方法や手段が実際に使われたものであり、結果は記録されたデータを正確に反映していることを確認した。

QAU 責任者

渡部 健

渡部 健

（株）日曹分析センター

2010年7月22日

Study Information

Study No.: NCAS 10-065

Study Title: Algal Growth Inhibition Study

Report No.: NCAS 10-065

Sponsor:

Test Facility: Nisso Chemical Analysis Service Co., Ltd.
Odawara Laboratory
345 Takada, Odawara, Kanagawa 250-0216, Japan
TEL 81-465-42-5268, FAX 81-465-42-3586

Study Director: Koji Baba
Experimenter: Chiho Nakamura (management of test organisms and all experimental procedures except analysis of test concentrations)
Koji Baba (first pre-culture [first definitive test])
Shinpei Oonuki, Chiaki Urabe (analysis of test concentrations)

Study Initiation Date: May 21, 2010
Experimental Start Date: May 25, 2010
Exposure Period: May 25, 2010 – May 28, 2010 (First definitive test, invalid)
June 7, 2010 – June 10, 2010 (Second definitive test, valid)
Experimental Completion Date: June 10, 2010
Study Completion Date: July 22, 2010

Test Guideline: Standard for the testing methods concerning new chemical substances. (Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, No.1121002, November 21, 2003; Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry, No.2, November 13, 2003; Environmental Policy Bureau, Ministry of the Environment, No. 031121002) The latest amendment: November 20, 2006, <Algal Growth Inhibition Test, Daphnia sp., Acute Immobilisation Test and Fish Acute toxicity test> IV Algal Growth Inhibition Test

Archiving: All the documents of this study will be retained in the archives of the test facility for 10 years after the completion of the study, but the place for storage after that will be decided on discussion with the sponsor. The test substance will be retained in the test facility at least for 10 years after the completion of the study, but only as long as the quality of the substance affords evaluation.

Deviations from the SOPs and the protocol: None

Circumstance / matter that may affect the reliability of the test results: None

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Summary

A 72-hour growth inhibition study (open system, shaking culture) was performed by exposing the algae to test solutions containing . A 24.0% aqueous solution of was used as a test substance and the concentrations of were expressed as itself unless otherwise specially stated. The measured concentrations of the test substance in the test solutions at 5 nominal concentrations of 6.25, 12.5, 25.0, 50.0 and 100 mg/L (common ratio: 2.0) were 104–106 % of the nominal at the start of the exposure and 99.6–103 % of the nominal at the end of the exposure. Since the variability of the measured concentrations was within $\pm 20\%$ of the nominal, the results were calculated on the basis of the nominal concentrations as the test concentrations during the exposure period. The concentration of the test substance that reduced algal growth by 50% (ErC₅₀) and the No Observed Effect Concentration (NOECr) values based on the growth rate for the 0-72-hour exposure period are presented below:

Exposure duration	ErC ₅₀ (mg/L)	95% confidence interval (mg/L)	NOECr (mg/L)
0-72 hour	> 100*	—	50.0

*: Since the % inhibition was under 50% even at the highest test concentration (100 mg/L), the ErC₅₀ was determined > 100 mg/L.

Introduction

This study was conducted in accordance with the following requirement to determine the 50% inhibition growth rate (ErC₅₀) and the No Observed Effect Concentration (NOECr) by exposing the algae to the test solutions containing .

- Standard for the testing methods concerning new chemical substances. (Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, No.1121002, November 21, 2003; Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry, No.2, November 13, 2003; Environmental Policy Bureau, Ministry of the Environment, No. 031121002) The latest amendment: November 20, 2006, <Algal Growth Inhibition Test, *Daphnia sp.*, Acute Immobilization Test and Fish Acute toxicity test> IV Algal Growth Inhibition Test

Materials and Methods

1. Test substance

Name:

Abbreviated name:

Product name:

Chemical name:

Structure:

CAS No.:

Molecular formula:

Molecular weight:

Lot No.:

NCAS retrieval No.: STD-1150

Purity: Solid content; 24.0% aqueous solution

Impurities: 0.85% (as against solid content), Cl ion; 35 ppm

In this report, the concentrations of the test substance were expressed as those of
unless otherwise specially stated. was defined as the solid contents
except water in this study.

Appearance:

Obtained from:

Received volume: 420 g

Received date: January 18, 2010

Expiration date: January 18, 2013

Storage conditions: Stored in a refrigerator with a polypropylene container

Water solubility: Soluble

Stability information: Stable in water

Summary of risk and harmfulness: Causes skin and eye irritation. Wear protective gloves, goggles and mask when it is used (from MSDS).

2. Verification of test substance

The identity of the test substance was verified by comparing the nuclear magnetic resonance spectrum (^{19}F -NMR) which was measured before the exposure initiation with another spectrum which was supplied by the sponsor. This verification was conducted in another study, NCAS 10-022 "Determination of the Concentration of in Dosing Solutions" and the result was utilized in this test.

3. Verification of stability of the test substance

The storage stability of was verified by confirming identity between the two nuclear magnetic

resonance spectra (^{19}F -NMR) which were measured before and after the exposure.

This verification was conducted in another study, NCAS 10-066

Acute Toxicity Study in *Oryzias latipes*” and the result was utilized in this study.

4. Verification of solubility of test substance

Since the test substance was a 24% aqueous solution, its water solubility was not measured.

5. Reagents and apparatus

Distilled water, acetonitrile: HPLC grade (Wako Pure Chemical Industries, Ltd.)

Formic acid: Special grade (Wako Pure Chemical Industries, Ltd.)

Ion exchange water: Tap water was distilled with a Barnstead water distilling apparatus (WDA-15S, Isuzu Seisakusho Co., Ltd.) and then purified with an ultrapure water system (LV-08, Toray Co., Ltd.), or purified with an ion exchange water system (SA2100E, Tokyo Rikakikai Co., Ltd.) to prepare ion exchange water.

Balances: AG285, AX205, AG204 and XP205 (Mettler Toledo)

Sterilization filters: Nylon membrane filter units, 0.2 μm (NALGENE)

pH meter: pH meter D-51 (HORIBA, Ltd.)

Illuminometer: LI-1400 (MEIWA FOSIS Co.)

Thermometer: Standard thermometer; measuring temperature range from 0 to 50°C, minimum scale 0.1 °C (Ando Keiki Co., Ltd.)

Incubator shaker: MR-100LS (Takasaki Scientific Instruments Corp.)

Clean bench: FG-1914L, BHC-1604 II A/B₃ (Airtech Japan, Ltd.)

Electronic particle counter: F-520P (Sysmex Corp.)

Electrolytic solution for the particle counter: Cellpuck (Sysmex Corp.)

Ultrasonic bath: UC-3 (Ikeda Scientific Co., Ltd.)

Microscope: BX51 TRF (Olympus Corp.)

Centrifuge: M200-IVD (Sakuma Co., Ltd.), KN-70 (Kubota Co.)

Micropipette: Eppendorf Research V (Eppendorf Co., Ltd.)

Centrifuge tube and test tube: 10-mL glass test tube with ground-in stopper

Liquid chromatograph mass spectrometer (LC/MS/MS): Acquity/Quattro micro API (Waters Corp.)

6. Test organisms

Taxonomic group: unicellular green algae

Scientific name: *Pseudokirchneriella subcapitata* (former name; *Selenastrum capricornutum*)

Obtained from: National Institute for Environmental Studies

Acquisition date: July 3, 2008

Strain: NIES-35

Sensitivity: The background data of the 0-72 hour ErC_{50} value of the reference substance (potassium dichromate) in *P. subcapitata* in our laboratory were

1.4 and 1.2 mg/L (n=2, conducted in June and December 2009, NCAS No.; 09-059).

Pre-culture conditions: The test organisms used in this study were cultured under the same conditions as those to be used in the study and in log growth phase at the start of the exposure. After the pre-culture period, the cells were observed microscopically to confirm that neither modification nor abnormal appearance of the algae was shown.

Culture period; June 4-7, 2010

Temperature; 23.1°C (in the incubator)

Lightning; 72-76 $\mu\text{E}/\text{m}^2/\text{s}$ under continuous illumination (cool white fluorescent lamp, measured at the level of the solutions in flasks)

Test room used: A4

7. Algal growth inhibition study

The definitive tests were conducted twice. Since variability of the growth rates in the control group was out of the criteria in the first definitive test, the test was not accepted. Accordingly, the second definitive test was conducted under the same conditions as the first definitive test except changing the rotation position of the test vessels in the incubator. In this report, only the second definitive test is described.

7.1 Test conditions

Type of culture:	Shaking culture (100 rpm)
Exposure period:	72 hours
Volume of test solutions:	100 mL / vessel
Vessels:	300-mL conical glass vessels with aluminum caps (air-permeable)
Replicates:	3 vessels / exposure group (6 vessels / control group)
Initial cell density:	5000 cells / mL (nominal)
Test temperature:	23±2°C
Lighting:	65–100 $\mu\text{E}/\text{m}^2/\text{s}$ under continuous illumination (cool white fluorescent lamp, measured at the level of the solutions in flasks)
pH:	The pH values of the test solution were not adjusted
Medium:	OECD medium (see Appendix 1)
Test room used:	A3

7.2 Selection of the concentrations of the test substance in the test solutions

A range-finding test (Report No.; NCAS 10-063NG) at nominal concentrations of 0.500, 5.00 and 50.0 mg/L (as was conducted and % inhibition of growth rates during 0-72 hours were 0, 0 and 4.95% respectively.

On the basis of the above results, the definitive test was conducted at 5 nominal concentrations (common ratio; 2.0 as LiFSI) of 6.25, 12.5, 25.0, 50.0 and 100 mg/L under open system.

7.3 Preparation of the test solutions

An amount of the test substance (624.9 mg; 150 mg as [corrected for purity (24.0%)]) was weighed and transferred to a 300-mL volumetric flask and dissolved in the OECD medium and the volume was adjusted to prepare a 500 mg/L stock solution. This stock solutions of 6.25, 12.5, 25.0 50.0 and 100 mL were separately transferred to 500-mL flasks and filled to volume with OECD medium to prepare the test solutions of 6.25, 12.5, 25.0, 50.0 and 100 mg/L.

Three vessels each containing 100 mL of the test medium were used for each exposure group. Six vessels each containing 100 mL of the OECD medium (without the test substance) were used for the control group.

7.4 Experimental procedures

The cell density in the pre-culture was 5.25×10^5 cells/mL. An amount of the pre-culture (952 μ L), which was calculated to provide the nominal cell density of 5000 cells/mL in each test solution, was aseptically introduced to each test vessel containing the test solution with a micropipette. After inoculation, the test vessels were placed in the incubator (nominal temperature of the incubator: 23.0°C) and the incubation started.

The cell density in each test vessels was measured after 24, 48 and 72 hours of the exposure .The test vessels were repositioned daily during the exposure period.

At the exposure initiation, pH in the remains of test solution was measured in each test group (each exposure and control group) after preparation. At the exposure termination, pH in one of the replicates was measured in each test group. Temperature and light intensity in the incubator were measured once daily during the exposure period.

Working contents were shown below in chronological order.

Exposure time (h)	Working content
0	Preparation of test solutions → Measurement of temperature and light intensity in the incubator → Measurement of the pre-culture cell density → Calculation of the inoculum volume → Exposure → Observation of test solutions, Measurement of pH, Microscopic observation of the cells in the pre-culture
24, 48	Measurement of cell density, Observation of test solutions, Measurement of temperature and light intensity in the incubator, Reposition of test vessels, Microscopic observation
72	Measurement of cell density, Observation of test solutions, Measurement of pH, Measurement of temperature and light intensity in the incubator, Microscopic observation

7.5 Method for measurement of the cell density of the test organism

The test solutions (or cultures) were diluted as the following table and the cell densities (cells/mL) of those were measured with the electronic particle counter. At exposure initiation, the nominal cell density (5000 cells/mL) in the test solution was used as an initial cell density.

The particle densities in the control and the highest concentration solutions without any algae were

1250 and 1180 counts/mL respectively. Since the measured values were lower than 7×10^4 counts/mL which was described in the protocol and sufficiently lower, the measured cell density with the electronic particle counter were recognized as cell density,

Measurement item	Aliquot of sample (μL)	Volume of cellpuck (μL)	Dilution factor
1. Cell density of the stock culture	100	9900	×100
2. Cell density of the pre-culture	200	9800	×50
3. Particle density of the OECD medium and the highest exposure solution (not containing algae)	5000	5000	×2
4. Cell density at 24 hours			
5. Cell density at 48 hours	200	9800	×50
6. Cell density at 72 hours			

7.6 Observations of the test solutions

The observations (color and precipitate) of the test solutions were conducted visually at the start and after 24, 48 and 72 hours of the exposure.

Microscopic observations of algae were conducted after 24, 48 and 72 hours of the exposure. An aliquot (1.5 mL) of the culture was collected into an eppendorf tube with a micropipette and centrifuged (nominal: 23 °C, 16500 rpm, 5 minutes). The supernatant was removed and the precipitation of alga was placed on a slide glass and observed microscopically. (1 vessel / group)

8. Analysis of the test concentrations

8.1 LC/MS/MS conditions

Apparatus:	Acquity / Quattro micro API (Waters)
Column:	Acquity UPLC BEH C18, 2.1 mm i.d. × 50 mm, particle diameter; 1.7 μm (Waters)
Mobile phase:	Acetonitrile + 0.1% formic acid aqueous solution (v/v) = 50 + 50 (v/v)
Flow rate:	0.3 mL/min
Column temperature:	40°C
Injection volume:	5 μL
Ionization mode:	ESI, Negative
Monitoring ion:	180.33 > 96.90 (quantification), 180.33 > 77.84 (confirmation)

8.2 Preparation of the calibration solutions and the calibration curves

An amount (5.05 mg) of the test substance was correctly weighed and transferred into a 50-mL volumetric flask and dissolved in 50% acetonitrile aqueous solution to prepare 101 mg/L calibration stock solution. This calibration stock solution was then further diluted with the same solvent to prepare 1.01, 2.02, 3.03, 4.04 and 5.05 mg/L calibration solutions. These calibration solutions were analyzed by using the LC/MC/MC conditions described in section 8.1. The Calibration curves were

generated by plotting the peak areas vs. the concentrations of and the linear regression equations and the correlation coefficients (r) were calculated by the least square method by using a computer program of LC/MS/MS (Mass Lynx v 4.1). The accuracy (%) for each calibration solution was calculated by inverse regression method. The weighting of $1/x$ was carried out. The standard solutions used to construct calibration curves were prepared before use.

8.3 Validation of the test substance concentration in the test solutions

8.3.1 Validation of the calibration curve

The accuracy was calculated by finding the regression equation and correlation coefficient (r) and then determining the quantitative value with inverse estimation method.

The criteria for the calibration curve were described below.

1. The correlation coefficient is more than 0.990.
2. The accuracy for the lowest concentration solution is within $\pm 20\%$, and that for other solutions is within $\pm 15\%$.

8.3.2 Validation of the repeatability

Analytical method was validated by the repeatability test without any recovery tests, because extraction was not conducted. The measured concentrations of the test substance in the highest and lowest concentration were calculated by the same procedure as described in section 8.4 in the first definitive test. The analytical method was validated by the coefficient of variations of the measured test concentrations ($n=3$) which were within $\pm 10\%$.

8.4 Measurement of the test substance concentration in the test solutions

The measurement was conducted at the exposure initiation and termination.

At the exposure initiation, 10-mL samples were collected from each remainder of the test solutions from each group after preparation. At the exposure termination, 3-mL samples were collected from each triplicate test solution from the mid-depth of the test vessels and the three samples from the same group were mixed to be a 9-mL sample solution. (1.5-mL samples were collected from each six replicate test solution in the control group).

The sample solutions were centrifuged (3000 rpm, 10 min) and the supernatants obtained were mixed with acetonitrile at the rate of 1:1 (v/v). These solutions were diluted with 50% acetonitrile aqueous solution to be an appropriate concentration that was in the middle of the range of the calibration curve, and then analyzed by using the LC/MS/MS conditions as described in section 8.1. The test substance concentration was calculated by substituting the peak area obtained into the calibration curve.

The sample solutions from the control group were also analyzed by the same procedures to verify that any interfering peak did not appear at the retention time of the test substance on the chromatogram.

Since the measured concentrations of the test substance at the start and end of the exposure were within $\pm 20\%$ of the nominal, the nominal concentrations were regarded as the test concentrations during the exposure period.

9. Calculation of the percent inhibition of growth rate

The growth curves (semilog graph) were prepared by plotting the time of measurement vs. the mean measured cell density in each test group (each exposure and control group). At this time, it was verified that the control culture was in log growth phase. Subsequently, the percent inhibition of growth rate was calculated as follows. The coefficient of variation for average specific growth rates and for section-by-section (daily) growth rates in replicate control cultures were calculated by using Microsoft® Excel 2003.

9.1 Calculation of the average specific growth rate (μ)

The average specific growth rate (μ) was calculated by using a natural logarithm:

$$\mu_1 = (\ln N_{24} - \ln N_0) / 24$$

$$\mu_2 = (\ln N_{48} - \ln N_{24}) / 24$$

$$\mu_3 = (\ln N_{72} - \ln N_{48}) / 24$$

$$\mu_4 = (\ln N_{72} - \ln N_0) / 72$$

where,

N_0 :	cell density at the start of the exposure (nominal)
N_{24} :	cell density at 24 hours
N_{48} :	cell density at 48 hours
N_{72} :	cell density at 72 hours
μ_1 :	average growth rate from 0 to 24 hours
μ_2 :	average growth rate from 24 to 48 hours
μ_3 :	average growth rate from 48 to 72 hours
μ_4 :	average growth rate from 0 to 72 hours

9.2 Calculation of the percent inhibition of growth rate (I_μ)

The percent inhibition of growth rate (I_μ) was calculated by comparison with the growth rates as follows:

$$I_\mu = (\mu_c - \mu_t) \times 100 / \mu_c$$

Where,

μ_c :	growth rate in the control group
μ_t :	growth rate in the exposure group

10. Calculation of 50% inhibition of growth rate (ErC_{50})

Since the percent inhibition of the growth rate was less than 50% even at the highest test concentration (100 mg/L), ErC_{50} (0-72 h) was not calculated statistically.

11. Determination of No Observed Effect Concentration (NOECr)

The NOECr was determined as the highest test concentration that caused no significant growth reduction. Based on the result of the Bartlett test ($\alpha=0.01$), homogeneity of variances was verified. After 1-way ANOVA ($\alpha=0.01, 0.05$), Dunnett test ($\alpha=0.01, 0.05, 2$ -sided) was conducted as a method of multiple comparison. NOECr was defined as the concentration of the highest exposure group which was not significantly different from the control group. All statistical analyses were performed by using the Yukms software of Statlight 2000 (Yukms Corp. Tokyo). The value of NOEC was indicated in 3-digit number.

Results

1. Validation of the test substance

The ^{19}F -NMR spectrum of which was supplied by the sponsor, is shown in Figure 1. The ^{19}F -NMR spectrum of the test substance, which was measured before the exposure initiation (conducted in NCAS No: 10-022 “Determination of the Concentration of in Dosing Solution”) is shown in Figure 2.

On the spectrum which was supplied by the sponsor, 3,3,4,4,5,5,6,6,7,7,8,8,8- Tridecafluoro-1-octanol was used as the chemical shift standard and its signal appeared at about 53 ppm. In this study, trifluoroacetic acid was used as the chemical shift standard and its signal appeared at about 51 ppm. It was considered that the difference in the chemical shift between the two spectrums was caused by using the different reference standard and the test substance was identified as

2. Validation of the stability of the test substance

The ^{19}F -NMR spectrum of the test substance, which was measured after the exposure termination (conducted in NCAS No: 10-066 : Acute toxicity test in *Oryzias latipes*”), is shown in Figure 3.

The stability during the storage period was verified by the consistency between the two spectrums which were measured at the start and end of the exposure.

3. Validation of the analytical methods

3.1 Validation of the calibration curve

A typical calibration curve is shown in Figure 4. A typical chromatogram of the standard solution (5.05 mg/L, at the start of the exposure) is shown in Figure 5.

Each correlation coefficient (r) of the calibration curves was more than 0.990 at the start and end of the exposure. The variations of the accuracy of the lowest standard samples were within $\pm 20\%$ and those of other standard samples were within $\pm 15\%$. Thus, it was determined that the linearity was acceptable and the calibration curves were validated.

3.2 Validation of the repeatability

The results of the repeatability test are shown in Table 1. The typical chromatogram of the repeatability test (nominal concentration: 100 mg/L) is shown in Figure 6.

The coefficient of variation ($n=3$) of the test concentrations were within 10%, therefore the analytical method for the determination of the concentration of the test substance in the test solution was determined as valid in this study.

4. Test concentrations of the test substance in the test solutions

The analytical results of the test substance in the test solutions are presented in Table 2. The chromatogram of the control group at the start of the exposure is shown in Figure 7, and chromatograms of 100 mg/L group at the start and end of the exposure are shown in Figure 8 and 9, respectively.

On the chromatogram of the control group, no interfering peak appeared at the retention time of the test substance.

The concentrations of the test substance in the test solutions were 104–106% of the nominal at the exposure initiation and 99.6 – 103% of the nominal at the exposure termination. The concentrations of the test substance in the test solutions were stable during the exposure period.

5. Growth curve and growth rate

The cell densities during the exposure period are presented in Table 3, the growth rates and the percent inhibitions of the growth rate are presented in Table 4 and the growth curves are presented in Figure 10. The cell density in the control cultures increased by a factor of 172 within the exposure of 72-hours which was satisfied the test criterion (more than 16-fold).

The mean coefficient of variation (CV) for daily specific growth rates (days 0–1, 1–2, 2–3) in the control cultures was 8.54% which was satisfied the criterion (within 35%). The coefficient of the variation of average specific growth rates during the whole exposure period (0-72 h) in replicate control cultures was 3.62% which was satisfied the criterion (within 7%). Since linearity of the growth curves in the control group was observed, it was confirmed that the cells were in the exponential growth phase during the exposure period.

6. ErC_{50} , 95% confidence interval and NOECr

Results of Dunnett test (2-sided) are presented in Table 4, the values of ErC_{50} , 95% confidence interval and NOECr are presented in Table 5, and results of percent inhibition of growth rate in each test concentration are shown in Figure 11.

The ErC_{50} (0-72 h) and NOECr were determined on the basis of the nominal test concentrations. Since the percent inhibition was less than 50% even at the highest concentration tested, the ErC_{50} (0-72h) was determined to be > 100 mg/L.

Based on the results of the Dunnett test (2-sided), the NOECr, which is defined as the highest concentration group that caused no significant different ($p \leq 0.05$) from the control data, was determined to be 50.0 mg/L.

7. Temperature and light intensity in the incubator

Measurements of temperature and light intensity in the incubator are presented in Table 6.

The temperatures ranged from 22.9 to 23.0°C which were within the acceptable range ($23 \pm 2^\circ\text{C}$).

Measurements of light intensity ranged from 69 to 86 $\mu\text{E}/\text{m}^2/\text{s}$ which were within the acceptable range ($65\text{--}100 \mu\text{E}/\text{m}^2/\text{s}$).

8. pH

The pH values of the test solutions are shown in Table 7.

The pH values of the control group at the start and end of the exposure were 8.0 and 7.9, respectively, and showed almost same value.

9. Observations of test solutions

The observations of the test solutions are presented in Table 8.

All test solutions were clear and colorless, and no coloration or precipitate due to the test substance was observed.

10. Microscopic observations of algae

The observations of the algae are presented in Table 9.

There were no morphological abnormalities in cell shape of the control cultures throughout the exposure period. Morphological abnormality (distorted shape) was observed in the 100 mg/L from 48 hours after the exposure.

11. Validity of the test

The validity of the test was confirmed because the following criteria were satisfied;

- The biomass (cell density) in the control cultures should have increased exponentially by a factor of at least 16 within 72 hours.
- The mean coefficient of variation for daily specific growth rates (days 0–1, 1–2, 2–3) in the control cultures must not exceed 35%.
- The coefficient of variation of average specific growth rates during the whole exposure period (0–3 day) in the control cultures must not exceed 7%.

Conclusions

The 72-hour growth inhibition study (open system, shaking culture) was performed by exposing the algae to the test solutions which contained It is concluded that the
0-72 hour ErC_{50} values is > 100 mg/L and correspondingly the No Observed Effect Concentration (NOECr) is 50.0 mg/L on the basis of the nominal concentrations.

Tables and Figures

Table 1 Result of validation of the analysis

Nominal concentration (mg/L)	Measured concentration (mg/L)	Mean measured concentration (mg/L)	Coefficient of variation (%)
6.25	6.04、6.02、6.10	6.05	0.688
100	98.5、98.0、99.0	98.5	0.508

Table 2 Concentration of test substance in test solution

Nominal concentration (mg/L)	Measured concentration (mg/L)	
	0 hour	72 hours
Control	—	—
6.25	6.60 (106)	6.34 (101)
12.5	13.0 (104)	12.7 (102)
25.0	26.2 (105)	25.4 (102)
50.0	52.3 (105)	49.8 (99.6)
100	106 (106)	103 (103)

() : Percent of nominal concentration (%)

Table 3 Cell density during the exposure period

Nominal concentration (mg/L)	Sample No.	Cell density (cells/mL)				Multiplication factor (0-72 h)
		0 h	24 h	48 h	72 h	
0.000 (Control)	1	5,000	26,100	159,000	782,000	
	2	5,000	23,700	162,000	1,040,000	
	3	5,000	24,700	116,000	622,000	
	4	5,000	25,800	164,000	978,000	
	5	5,000	21,500	147,000	817,000	
	6	5,000	24,300	165,000	922,000	
	Average	5,000	24,350	152,167	860,167	172
	SD	— ^{*1}	1,663	18,883	151,489	—
6.25	1	5,000	24,700	176,000	1,090,000	
	2	5,000	22,700	135,000	584,000	
	3	5,000	23,900	160,000	1,160,000	
	Average	5,000	23,767	157,000	944,667	189
	SD	— ^{*1}	1,007	20,664	314,301	—
12.5	1	5,000	27,300	168,000	1,360,000	
	2	5,000	23,900	180,000	1,420,000	
	3	5,000	26,700	179,000	963,000	
	Average	5,000	25,967	175,667	1,247,667	250
	SD	— ^{*1}	1,815	6,658	248,347	—
25.0	1	5,000	29,000	175,000	1,090,000	
	2	5,000	21,900	181,000	902,000	
	3	5,000	25,200	182,000	1,110,000	
	Average	5,000	25,367	179,333	1,034,000	207
	SD	— ^{*1}	3,553	3,786	114,752	—
50.0	1	5,000	22,700	146,000	971,000	
	2	5,000	25,700	155,000	799,000	
	3	5,000	21,400	157,000	915,000	
	Average	5,000	23,267	152,667	895,000	179
	SD	— ^{*1}	2,205	5,859	87,727	—
100	1	5,000	17,900	88,900	329,000	
	2	5,000	19,200	80,700	261,000	
	3	5,000	19,400	57,000	124,000	
	Average	5,000	18,833	75,533	238,000	48
	SD	— ^{*1}	814	16,566	104,417	—

*1 : SD was not calculated since the cell density at 0 hour was nominal initial density.

Table 4 Growth rate and percent inhibition of growth rate

Nominal concentration (mg/L)	Sample No.	Growth rate μ_1 (0-24 h)	Growth rate μ_2 (24-48 h)	Growth rate μ_3 (48-72 h)	Growth rate $\bar{\mu}_4$ (0-72 h)	% Inhibition I_{μ_4} (%)	Growth rate in control group (μ_1, μ_2, μ_3)		
							Average	SD	CV (%)
0.000 (Control)	1	0.0689	0.0753	0.0664	0.0702	—	0.0702	0.00459	6.54
	2	0.0648	0.0801	0.0775	0.0741	—	0.0741	0.00819	11.1
	3	0.0666	0.0644	0.0700	0.0670	—	0.0670	0.00282	4.21
	4	0.0684	0.0771	0.0744	0.0733	—	0.0733	0.00445	6.07
	5	0.0608	0.0801	0.0715	0.0708	—	0.0708	0.00967	13.7
	6	0.0659	0.0798	0.0717	0.0725	—	0.0725	0.00698	9.63
	Average	0.0659	0.0761	0.0719	0.0713	—	—	—	8.54
	SD	0.00293	0.00607	0.00379	0.00258	—			
	CV (%)	4.45	7.98	5.27	3.62	—			
6.25	1	0.0666	0.0818	0.0760	0.0748	-4.91			
	2	0.0630	0.0743	0.0610	0.0661	7.29			
	3	0.0652	0.0792	0.0825	0.0756	-6.03			
	Average	0.0649	0.0784	0.0732	0.0722 ^{n.s.}	-1.22			
	SD	0.00181	0.00381	0.0110	0.00527	—			
12.5	1	0.0707	0.0757	0.0871	0.0779	-9.26			
	2	0.0652	0.0841	0.0861	0.0785	-10.1			
	3	0.0698	0.0793	0.0701	0.0731	-2.52			
	Average	0.0686	0.0797	0.0811	0.0765 ^{n.s.}	-7.29			
	SD	0.00295	0.00421	0.00954	0.00296	—			
25.0	1	0.0732	0.0749	0.0762	0.0748	-4.91			
	2	0.0615	0.0880	0.0669	0.0722	-1.26			
	3	0.0674	0.0824	0.0753	0.0750	-5.19			
	Average	0.0674	0.0818	0.0728	0.0740 ^{n.s.}	-3.79			
	SD	0.00585	0.00657	0.00513	0.00156	—			
50.0	1	0.0630	0.0776	0.0789	0.0732	-2.66			
	2	0.0682	0.0749	0.0683	0.0705	1.12			
	3	0.0606	0.0830	0.0734	0.0724	-1.54			
	Average	0.0639	0.0785	0.0735	0.0720 ^{n.s.}	-1.03			
	SD	0.00389	0.00412	0.00530	0.00139	—			
100	1	0.0531	0.0668	0.0545	0.0581	18.5			
	2	0.0561	0.0598	0.0490	0.0549	23.0			
	3	0.0565	0.0449	0.0324	0.0446	37.4			
	Average	0.0552	0.0572	0.0453	0.0525 *	26.3			
	SD	0.00186	0.0112	0.0115	0.00705	—			

Results of Dunnett test (2-sided)

*: Indicates a significant difference from the control group (P<0.05)

n.s.: Indicates no significant difference from the control group

Table 5 ErC_{50} , 95% confidence interval and NOECr

Exposure duration	ErC_{50} (mg/L)	95% confidence interval (mg/L)	NOECr (mg/L)
0–72 hour	> 100*	—	50.0

* : Since the inhibition was under 50% even at the highest test concentration, the ErC_{50} was determined >100 mg/L.

Table 6 Temperature and light intensity in the incubator

Parameter	Measurement position	Exposure duration (h)				Range
		0	24*	48*	72	
Temperature (°C)	—	23.0	23.0	23.0	22.9	22.9–23.0
Light intensity ($\mu E/m^2/s$)	No.1	72	—	—	73	69–86
	No.2	70	71	73	73	
	No.3	84	—	—	86	
	No.4	70	—	—	74	
	No.5	69	—	—	72	

*: Light intensity was measured at only No.2 position.

Table 7 pH of the test solutions

Nominal concentration (mg/L)	pH	
	0 hour	72 hours
Control	8.0	7.9
6.25	8.0	7.9
12.5	8.0	8.0
25.0	8.0	8.0
50.0	8.1	7.9
100	8.1	7.9

Table 8 Observations of the test solutions

Nominal Concentration (mg/L)	Exposure duration			
	0 hour	24 hours	48 hours	72 hours
Control	Colorless and transparent	Colorless and transparent	Slightly green	Green
6.25	Colorless and transparent	Colorless and transparent	Slightly green	Green
12.5	Colorless and transparent	Colorless and transparent	Slightly green	Green
25.0	Colorless and transparent	Colorless and transparent	Slightly green	Green
50.0	Colorless and transparent	Colorless and transparent	Slightly green	Green
100	Colorless and transparent	Colorless and transparent	Colorless and transparent	Slightly green

Table 9 Microscopic observations of algae

Nominal concentration (mg/L)	Exposure duration		
	24 hours	48 hours	72 hours
Control	Normal	Normal	Normal
6.25	Normal	Normal	Normal
12.5	Normal	Normal	Normal
25.0	Normal	Normal	Normal
50.0	Normal	Normal	Normal
100	Normal	Few distorted shape was observed	Distorted shape was observed

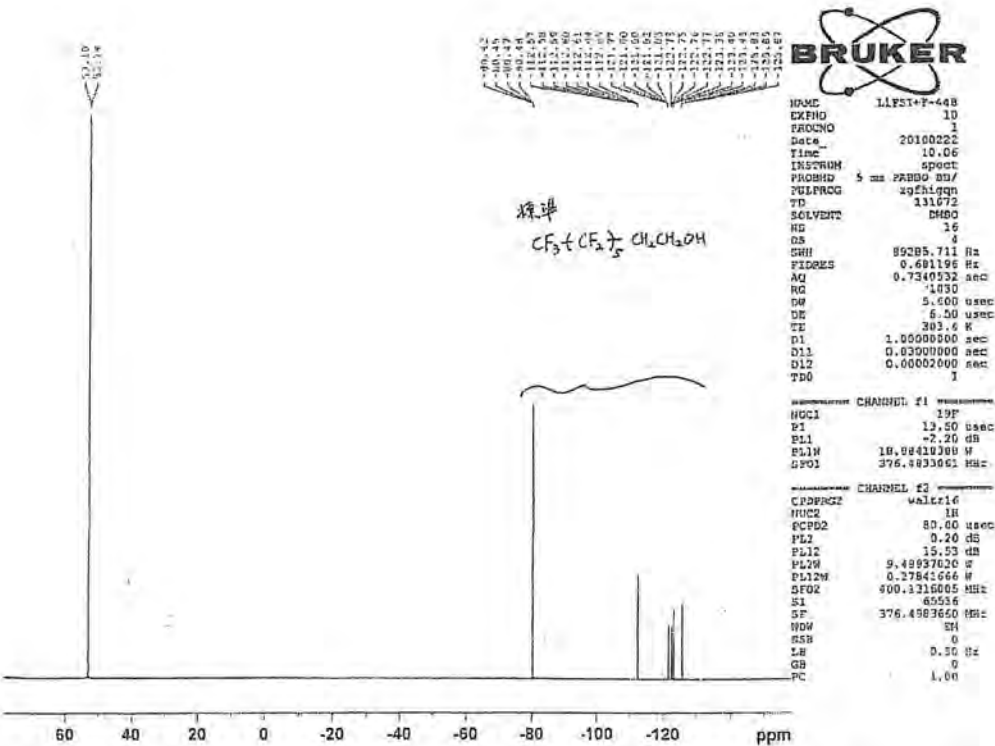


Figure 1 NMR spectrum of (supplied by Sponsor, ¹⁹F-NMR)

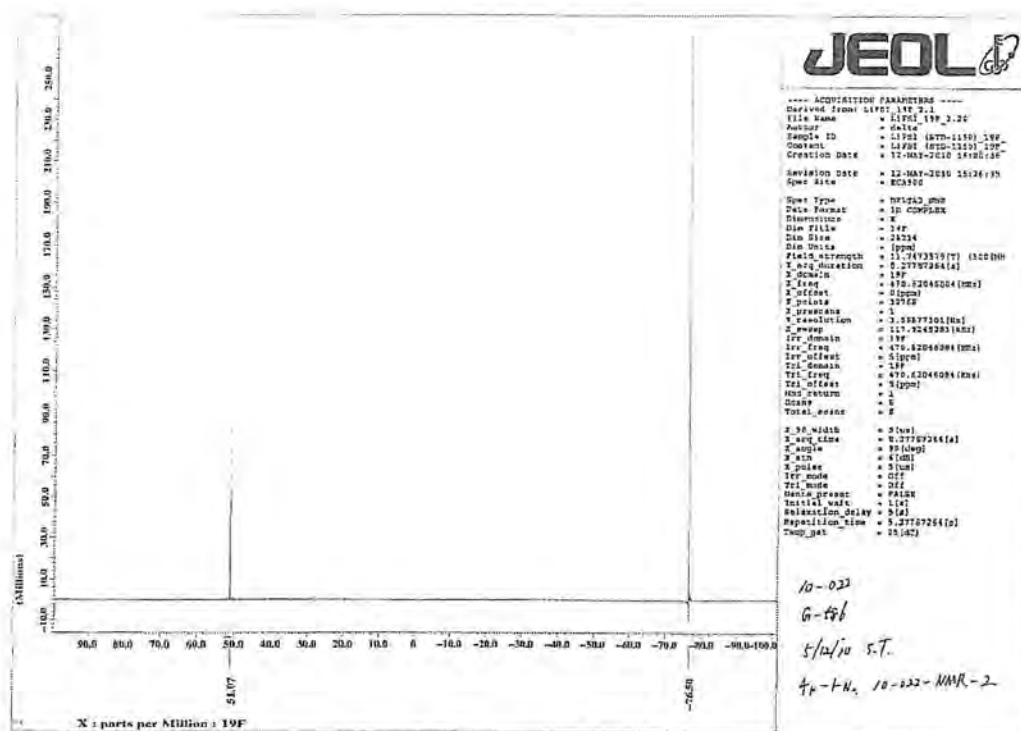


Figure 2 NMR spectrum of the test substance
(Before the exposure initiation, Study No.: NCAS 10-022)

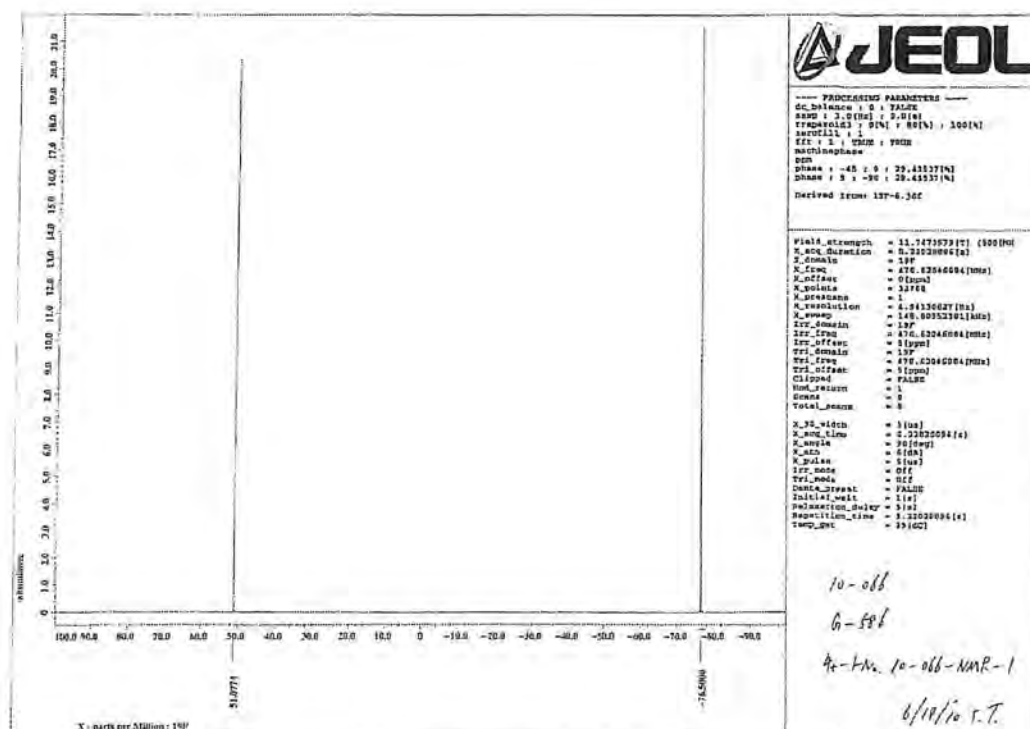


Figure 3 NMR spectrum of the test substance
(After the exposure termination, Study No.: NCAS 10-066)

Concentration (mg/L)	Peak area	Quantitative value (mg/L)	accuracy (%)
1.01	1894	0.948	93.8
2.02	3603	2.14	106
3.03	5041	3.13	103
4.04	6388	4.07	101
5.05	7527	4.86	96.3

Calibration: 07 Jun 2010 16:22:04

Compound name:

Correlation coefficient: $r = 0.997416$, $r^2 = 0.994838$

Calibration curve: $1438.82 * x + 530.659$

Response type: External Std, Area

Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

定量用

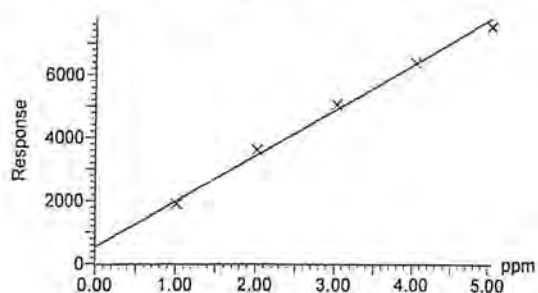


Figure 4 Typical calibration curve

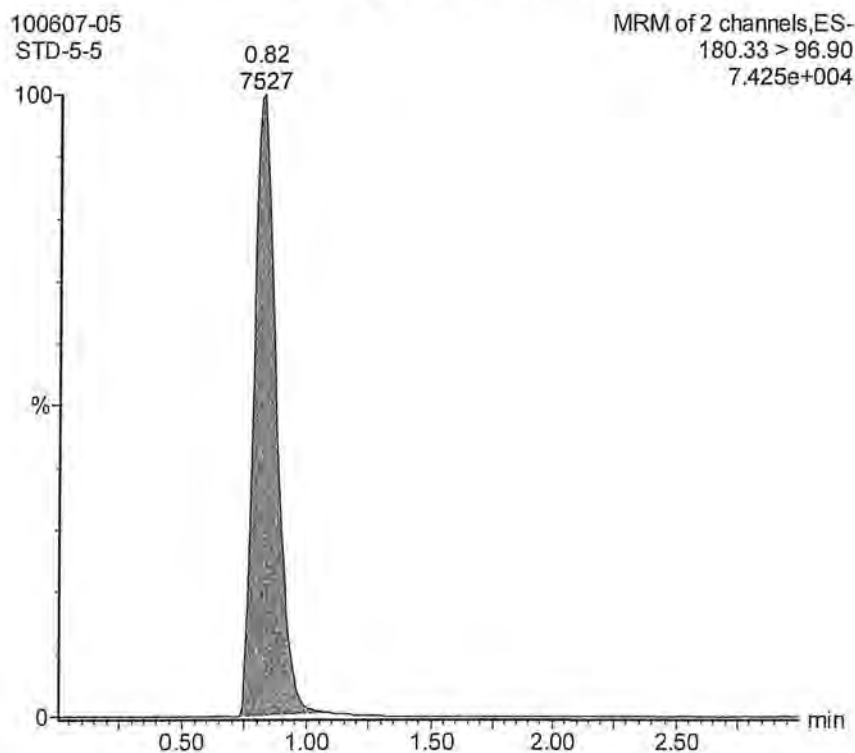


Figure 5 Typical chromatogram of a standard solution
(5.05 mg/L, at the start of the exposure)

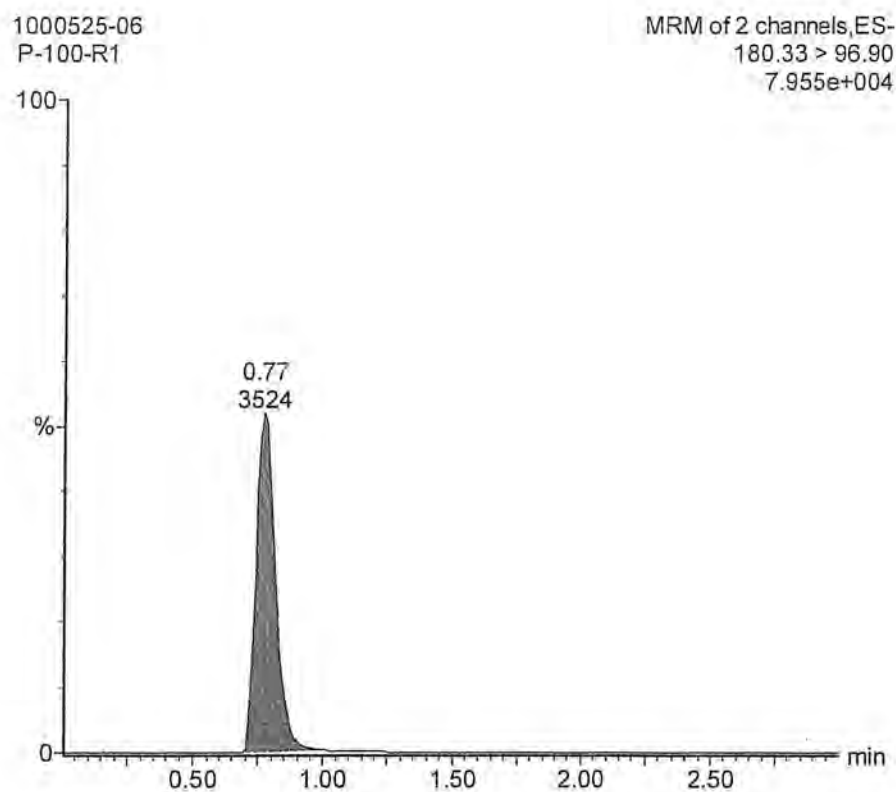


Figure 6 Typical chromatogram of a repeatability test sample

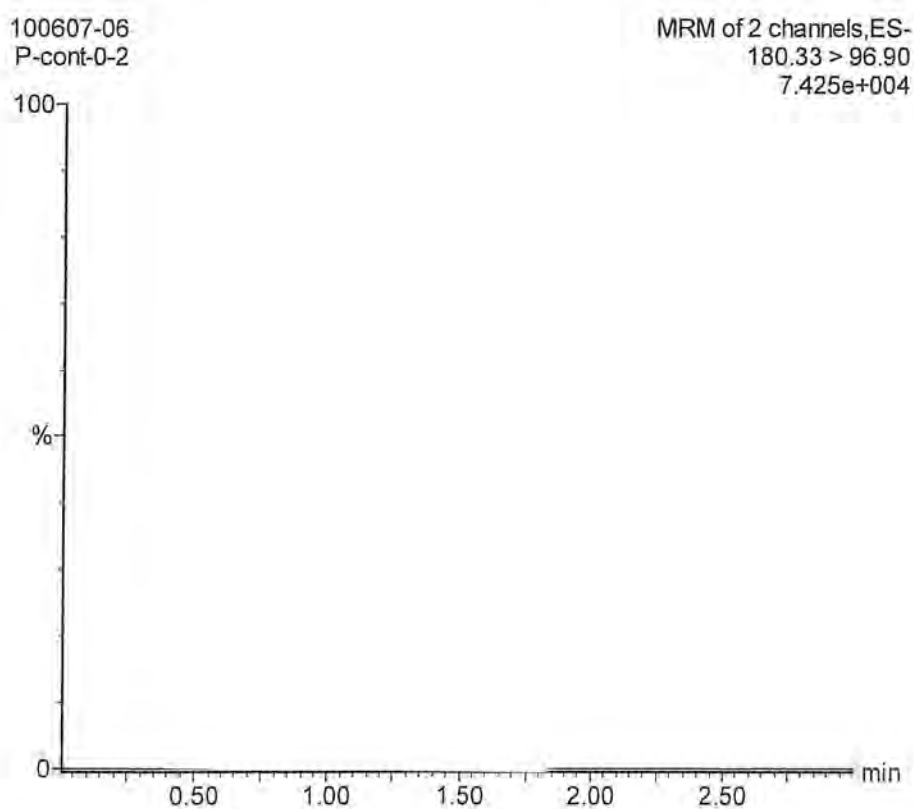


Figure 7 Chromatogram of the control group (at the start of the exposure)

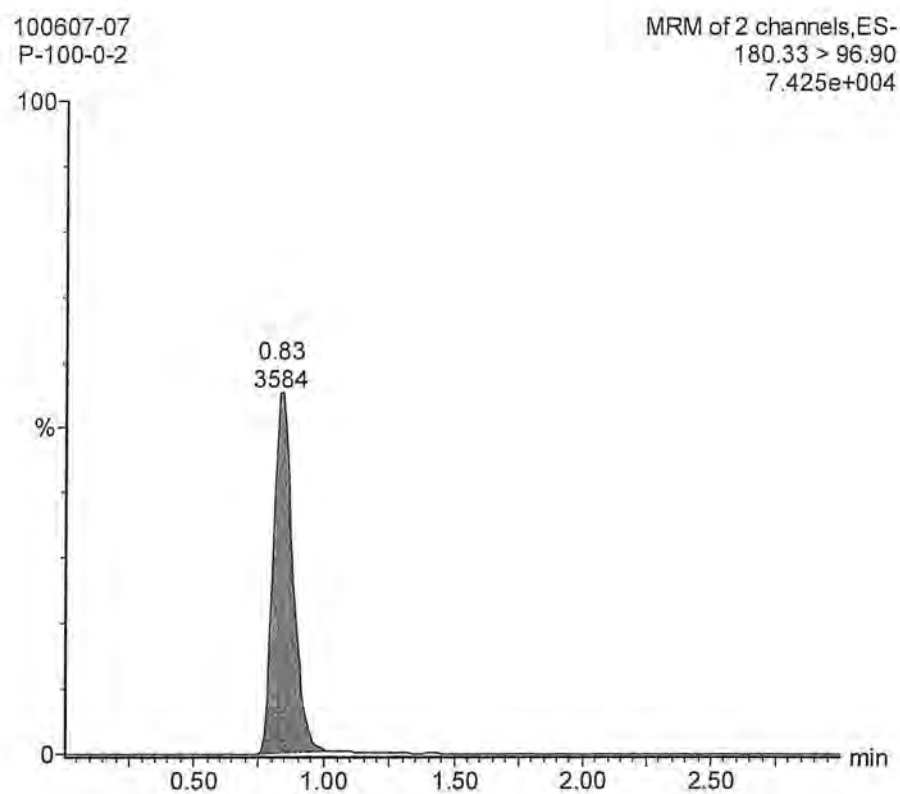


Figure 8 Chromatogram of the 100 mg/L group (at the start of the exposure)

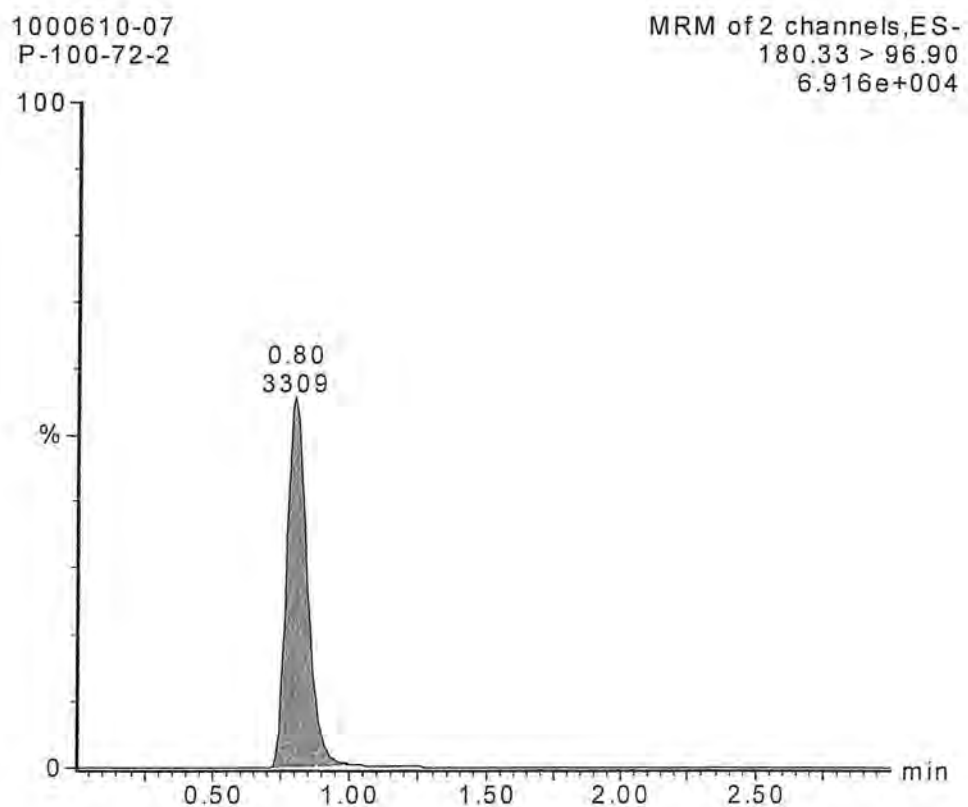


Figure 9 Chromatogram of the 100 mg/L group (at the end of the exposure)

Nominal concentration (mg/L)	Mean cell density (cells/mL)			
	0 hour	24 hours	48 hours	72 hours
0.000 (Control)	5,000	24,350	152,167	860,167
6.25	5,000	23,767	157,000	944,667
12.5	5,000	25,967	175,667	1,247,667
25.0	5,000	25,367	179,333	1,034,000
50.0	5,000	23,267	152,667	895,000
100	5,000	18,833	75,533	238,000

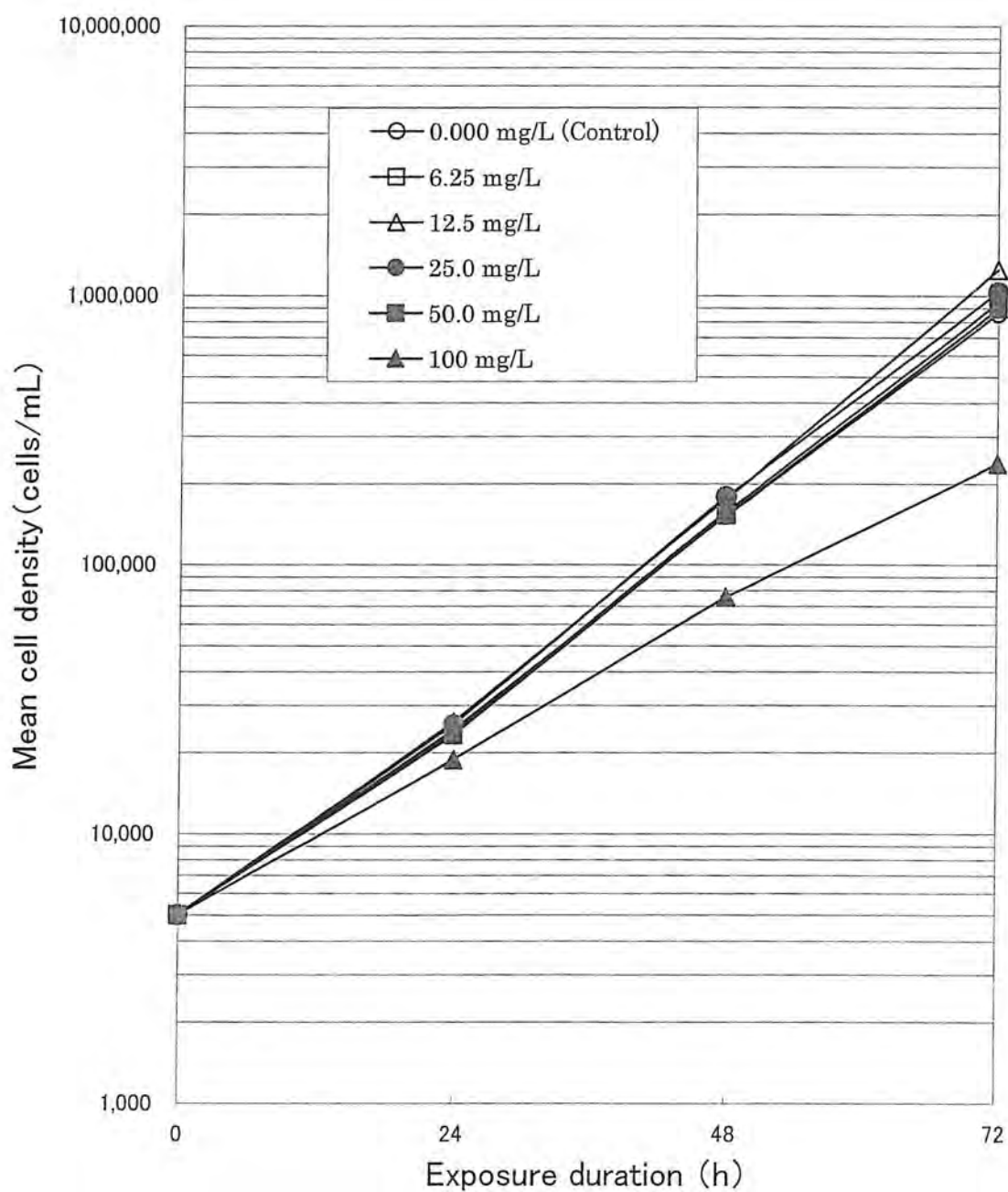


Figure 10 Growth curves of algae

Nominal concentration (mg/L)	Inhibition of growth rate (%)
	0-72 h
6.25	0.00*
12.5	0.00*
25.0	0.00*
50.0	0.00*
100	26.3

*: Values were regarded as 0.00 since % inhibition was <0

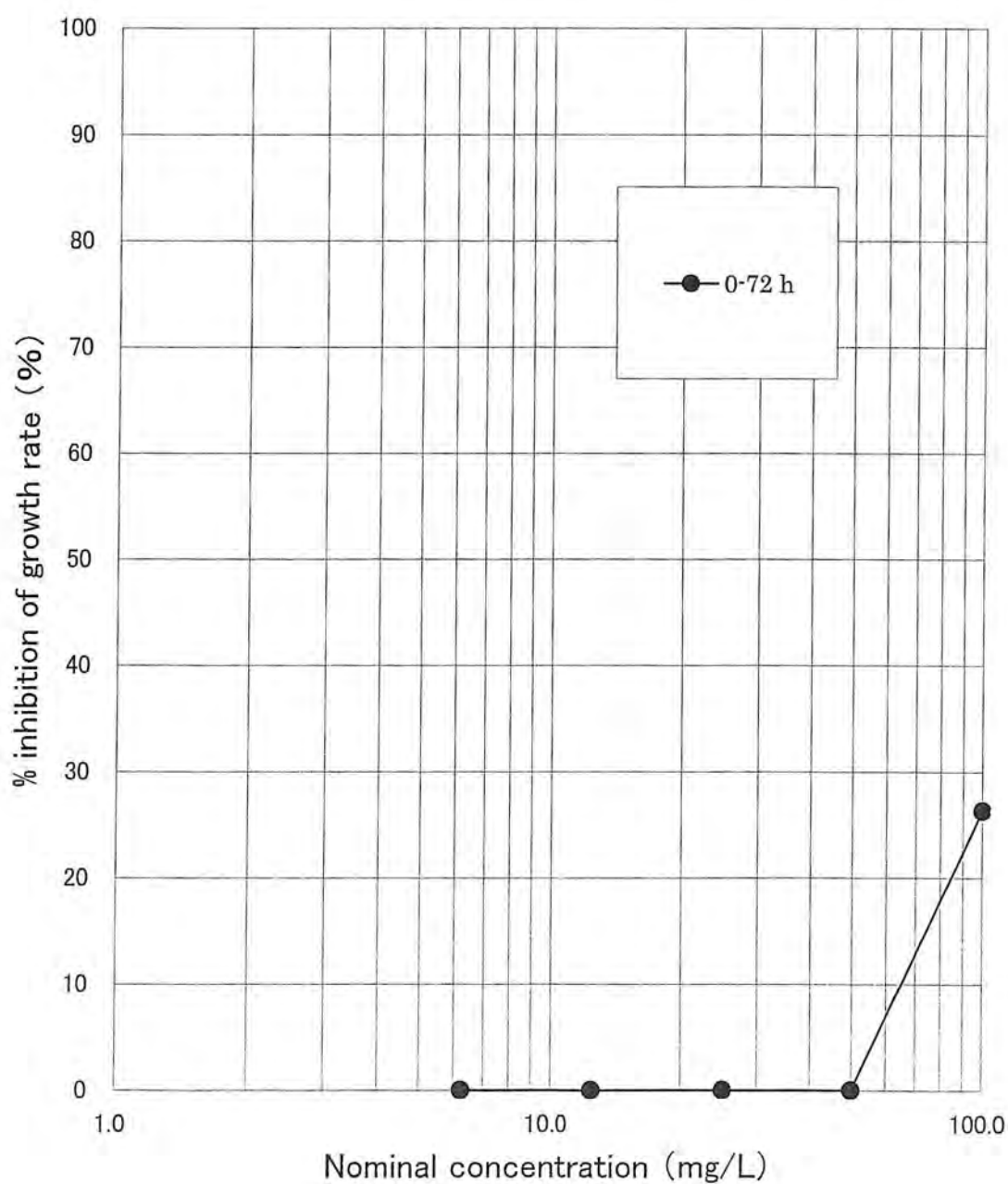


Figure 11 Percentage inhibition of growth rate calculated for each test group

Appendix 1 OECD medium

Preparation of Algal Medium

- A series of stock solutions of A (10 mL), B (1.0 mL) and C (1.0 mL) which had been sterilized with a filter (0.2 μm) were transferred to a 1-L volumetric flask in order and the volume was adjusted with Ion exchange water and then sterilized with the filter again to prepare the algal culture medium.
- The pH of the medium after preparation was confirmed to be within 8.1 ± 0.2 by measurements.

Stock solution	Reagent	Final concentration in test solution (mg/L)
A	NH_4Cl	15
	$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	12
	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	18
	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	15
	KH_2PO_4	1.6
B	$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	0.064
	$\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$	0.1
C	H_3BO_3	0.185
	$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.415
	ZnCl_2	0.003
	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.0015
	$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	0.00001
	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.007
D	NaHCO_3	50

All reagents used were special grade, obtained from Wako Pure Chemical Industries, Ltd.

All solutions described above were diluted with ion exchange water.

Authenticity of Translation

I declare that the original Japanese final report (Report No. NCAS 10-065) is translated into English consistently.

Translated by :



Osamu Saika

Nisso Chemical Analysis Service Co., Ltd.

Odawara Laboratory

June 5, 2012